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Short Report: Quantification of *Plasmodium falciparum* Gametocytes by Magnetic Fractionation

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Abstract. A method of gametocyte quantitation in human blood was developed based on magnetic fractionation using commercially available magnetic fractionation columns (MFCs) and exploiting the magnetic susceptibility of mature *Plasmodium falciparum* gametocytes. The technique uses magnetic microspheres as a calibration standard. Microspheres are added to each blood sample to a known concentration. When exposed to a magnetic field, gametocytes and magnetic microspheres are preferentially captured inside MFCs. After removal of the magnetizing field, the magnetically captured material can be eluted, placed on a microscope slide that is stained, and counted by using conventional methods. The limits of quantitation for *P. falciparum* gametocytes were determined from serial dilutions of blood samples with known gametocyte density. The upper limit was 1,000 gametocytes/μL. Quantitative analysis above this threshold is difficult because of an over-abundance of gametocytes. The lower limit was 0.1 gametocytes/μL, and there is a significant probability of a false-negative result below this level.

Plasmodium falciparum gametocytes can be detected in human blood by microscopic examination of blood films, but molecular techniques and high field gradient magnetic fractionation (HFGMF) have recently been developed as more sensitive alternative methods.[1,2] Although non-quantitative detection emphasizes the need for gametocidal therapy in clinical practice, an accurate estimate of submicroscopic gametocyte densities by using techniques such as HFGMF is important in epidemiologic studies because densities above the threshold of mosquito infectivity can play a major role in malaria transmission.[3]

In standard blood film quantitation by microscopy, asexual parasites and gametocytes are counted with reference to numbers of erythrocytes (thin films) or leukocytes (thick films), and the respective ratios are converted to a parasite or gametocyte density by using the erythrocyte and leukocyte densities, respectively. However, in the process of concentrating asexual forms and gametocytes by exploitation of their magnetic properties, the HFGMF process distorts these ratios relative to those in the original blood sample.[2] Therefore, there is a need for a simple and cost-effective way of correcting for this distortion so that HFGMF can generate valid gametocyte densities. In the present study, superparamagnetic microspheres of a few micrometers in diameter were used as a calibration standard in the measurement of submicroscopic gametocyte densities in the range of previously described quantitative molecular approaches.[4]

Gametocytes were obtained by culturing *P. falciparum in vitro* according to the standard methods described by Trager and Jensen with slight modifications.[5] To mimic blood samples with a wide variety of gametocyte densities, 10-fold serial dilutions of gametocytes were prepared in fresh human whole blood in eight separate experiments. The blood was from the same donor who provided the erythrocytes used in the *in vitro* cultures. Thick blood films were prepared from the samples with the most gametocytes. The gametocyte density in these thick blood films was determined by counting the number of gametocytes in 1,000 leukocytes and assuming, by convention, that 1 μL of blood contains 8,000 leukocytes.[6] The gametocyte densities in these thick blood films were in the range 1,000–10,000 cells/μL and served as the basis for the calculation of densities in the higher dilutions. The highest dilutions contained between 0.01 and 0.1 gametocytes/μL of blood.

High field gradient magnetic fractionation was conducted by using standard 400-μL samples from the serial dilutions and modifications of previously reported methods.[2] The first modification was that magnetic microspheres (fluorescent carboxyl magnetic particles, PAK blue, 7.0–7.9 μm diameter; Spherotech Inc., Lake Forrest, IL) were added to the sample before passage through the HFGMF columns. The volume of magnetic microspheres required to achieve a final concentration of 100 microspheres/μL was determined by using a hemocytometer. Second, the magnetically positive fraction was not fixed with methanol but only stained with Giemsa (5% v/v for 30 min) after it had been applied to a microscope slide in a similar way to the preparation of a thick blood film. This protocol resulted in a better contrast between magnetic microspheres and the background of erythrocyte ghosts.

The magnetic microspheres do not bind as tightly as erythrocytes to the glass slide. To avoid microspheres being washed off, the slides were not rinsed under running tap water but were dipped carefully into a beaker containing water to remove excess stain. Using this method, we determined that 90% (SD = 9.0%) of microspheres remain on the slide. Gametocytes and magnetic microspheres exhibited similar spatial distributions on the blood films. However, the ratio of gametocytes to magnetic microspheres close to the edges of some of the films was significantly higher than in the center of the film. To avoid possible bias caused by this edge effect, microspheres and gametocytes were counted in a standardized way from one edge of the film through the center to the other edge.

Gametocyte density was determined by calculating the ratio of gametocytes to magnetic microspheres on the slides. An area of the film containing 100 superparamagnetic microspheres was assumed to correspond to 1 μL of original blood volume. Because of the wide range of gametocyte densities, the volume of blood that was analyzed on the slides in the present study ranged from 1 μL to 20 μL (100–2,000 microspheres) depending on the gametocyte density.

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The appearance of the slides prepared after HFGMF is shown in Figure 1. Magnetic microspheres and gametocytes can be clearly identified. Above 1,000 gametocytes/μL, the number of gametocytes on the slides after HFGMF was too high to be counted accurately because there were hundreds of gametocytes in each field of view. Therefore, these slides were discarded. The correlation between known gametocyte densities between 0.01 and 1,000 gametocytes/μL and the respective gametocyte densities determined by quantitative HFGMF is shown in Figure 2A. A Bland-Altman plot of the same data is shown in Figure 2B. For known gametocyte densities greater than 0.1 gametocytes/μL, the association is linear with a correlation coefficient (r^2) of 0.95 and a mean ± SD slope of 0.98 ± 0.04. From these data, it is clear that gametocytes and microspheres are captured by, and released from, the magnetic fractionation columns with similar efficiency and that the use of the microspheres enables valid quantitative gametocyte density estimates. Below 0.1 gametocytes/μL, the line curves towards the y-axis because the number of gametocytes in the sample is so low that there is a significant chance that no gametocyte will be detected.[2] This situation leads to either an overestimation of the gametocyte density if one or two gametocytes are observed or a false-negative result if no gametocytes are observed.

The present method enables simple and quantitative measurement of gametocyte densities down to 0.1 gametocytes/μL in dilutions of human blood. In comparison, molecular methods have been reported to exhibit sensitivities of detection between 0.002 and 2 gametocytes/μL depending on the method and the blood volume used.[4,7,8] In HFGMF, the quantitative detection limit is determined by the chosen concentration of microspheres and the volume of blood. In the present study, we chose 100 microspheres/μL and counted up to 2,000 microspheres, which represented a maximum volume of blood of approximately 20 μL on the slide. The technique does not add significant expense to conventional methods because

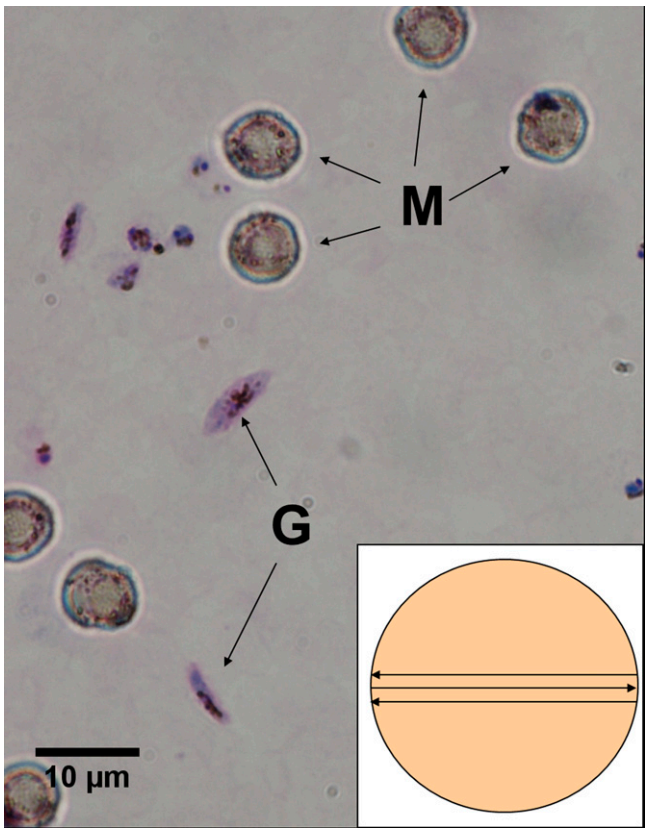

FIGURE 1. Gametocytes (**G**) and superparamagnetic microspheres (**M**) on a slide after high field gradient magnetic fractionation. Overall gametocyte density on this slide was 75 gametocytes/μL. **Inset** shows a schematic of the film shape, and **arrows** indicate the counting pattern. This figure appears in color at www.ajtmh.org.

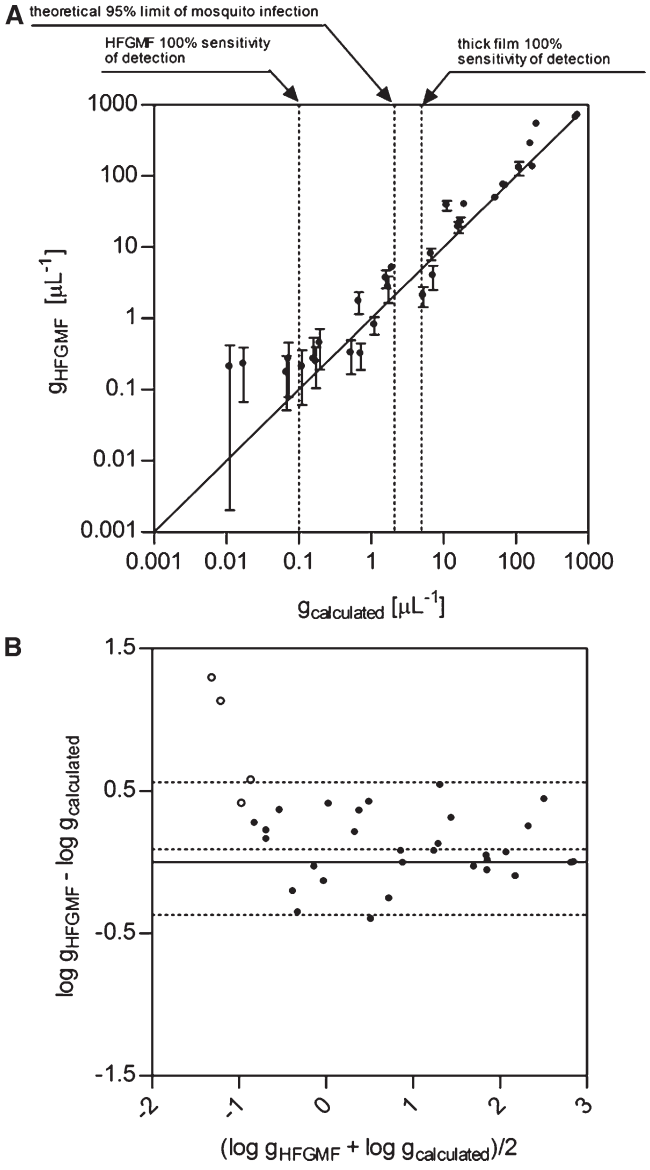

FIGURE 2. **A**, Correlation of known gametocyte density and gametocyte density obtained by high field gradient magnetic fractionation (HFGMF) in the range between 0.01 and 1,000 gametocytes/μL. The limit of 100% sensitivity of detection at 0.1 gametocytes/μL marks the threshold for a significant probability of false-negative results. Values below this cut-off point were excluded from linear regression analysis. Error bars are deduced from the Poisson statistics of counting. **B**, Bland-Altman plot of the logarithmically transformed gametocyte density values. The inner dotted line is the mean difference between known and HFGMF gametocyte density. The outer dotted lines are the upper and lower 95% limits of agreement between known gametocyte density and the one measured by HFGMF. The empty circles correspond to values below the 0.1 gametocyte/μL cut-off point and were excluded from Bland-Altman analysis.

the superparamagnetic spheres used in this study cost 8.5 US cents/μL and only 6.4 μL were used for each test.

Although the present study addresses the specific issue of *P. falciparum* gametocyte quantitation, our novel HFGMF method could potentially determine densities of trophozoites, schizonts, and gametocytes of non-*P. falciparum* species, and hemozoin-containing leukocytes.

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REFERENCES

1. Karl S, David M, Moore L, Grimberg BT, Michon P, Mueller I, Zborowski M, Zimmerman PA, 2008. Enhanced detection of gametocytes by magnetic deposition microscopy predicts higher potential for *Plasmodium falciparum* transmission. *Malar J 7:* 66.
2. Karl S, Davis TM, St.-Pierre TG, 2009. A comparison of the sensitivities of detection of *Plasmodium falciparum* gametocytes by magnetic fractionation, thick blood film microscopy, and RT-PCR. *Malar J 8:* 98.
3. Schneider P, Bousema JT, Gouagna LC, Otieno S, van de Vegte-Bolmer M, Omar SA, Sauerwein RW, 2007. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg 76:* 470–474.
4. Schneider P, Schoone G, Schallig H, Verhage D, Telgt D, Eling W, Sauerwein R, 2004. Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol 137:* 35–41.
5. Trager W, Jensen JB, 1976. Human malaria parasites in continuous culture. *Science 193:* 673–675.
6. McKenzie FE, Prudhomme WA, Magill AJ, Forney JR, Permpanich B, Lucas C, Gasser RA Jr, Wongsrichanalai C, 2005. White blood cell counts and malaria. *J Infect Dis 192:* 323–330.
7. Babiker HA, Abdel-Wahab A, Ahmed S, Suleiman S, Ranford-Cartwright L, Carter R, Walliker D, 1999. Detection of low level *Plasmodium falciparum* gametocytes using reverse transcriptase polymerase chain reaction. *Mol Biochem Parasitol 99:* 143–148.
8. Buates S, Bantuchai S, Sattabongkot J, Han ET, Tsuboi T, Udomsangpetch R, Sirichaisinthop J, Tan-ariya P, 2010. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes. *Parasitol Int 59:* 414–420.